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RESEARCH ARTICLE

Comparing the Neuroprotective Effects of Aqueous and Methanolic Extracts of *Vernonia Amygdalina* on the Cerebellum of Adult Male Wistar Rats



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ABSTRACT

The solvent of extraction determines the type and amount of phytochemicals extractable from a plant material. Reports have shown that numerous African herbs are rich in potent phytochemicals which have been used in folkloric and orthodox medicine in the prevention and treatment of ailments. This research is geared towards comparing the neuroprotective potency of the aqueous and methanolic extracts of *Vernonia amygdalina* on the cerebellum of mercury chloride intoxicated Wistar rats. Thirty adult male Wistar rats were divided into 6 groups of 5 rats each. Group A was the negative control and had food and water only. Group B was the positive control and was exposed to mercury chloride without treatment. Groups C1 and C2 received 200mg/kg bw of aqueous and methanolic extracts respectively while groups D1 and D2 received 400mg/kg bw of aqueous and methanolic extracts of *Vernonia amygdalina* respectively. Result of phytochemical analysis shows that water extracted more flavonoids while methanol extracted more phenols. The results of antioxidant studies show that the methanolic extract conferred more protection against oxidative stress than the aqueous extract. Histological results show that aqueous extract conferred more protection than the methanolic extract. We therefore conclude from our report that both the aqueous and methanolic extracts of *Vernonia amygdalina* confer neuroprotection on the cerebellum of Wistar rats.

Keywords: Methanol, Aqueous, Neuroprotective, Cerebellum, *Vernonia amygdalina*, Antioxidant

1 Introduction

Since the later part of the last millennium, there has been a growing interest in the screening of medicinal plants for their therapeutic properties and their possible use in combating ailments across the globe. This is however not new to Africa as herbs and plants remained a major source of treatment and cure of medical conditions by herbalists and native doctors. With the medical and technological advancement today, these plants are now screened for the presence of bioactive compounds which are known to possess antioxidant and other therapeutic properties [1]. Parts of these plants

like the leaves, bark and roots are very rich in phytochemicals such as phenols, alkaloids, flavonoids, terpenes, glycosides with the inherent capacity to mitigate diseases and attenuate toxicities [2]. Several medicinal plants are of much interest due to their antioxidant and free radical scavenging properties.

Oxidative stress has been associated with the aetiology of several ailments across the globe. Oxidative stress itself is induced by free radicals released into the body in the course of metabolic activities. Antioxidants on the other hand are electron sufficient compounds with the readiness to donate electron to electron-deficient free



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radicals to stabilize their actions. Free radicals are electron-deficient compounds because they possess unpaired valence electrons [3] and in their quest to finding electrons, they can easily attack cells and biomolecules in the body resulting in generation of diseases. Several studies has linked the generation of reactive oxygen species (free radicals) such as hydroxyl (OHG) radical, superoxide (O_2^-), nitric oxide (NO), nitrogen dioxide (NO_2), peroxy (ROOG), hydrogen peroxide (H_2O_2) to the development of pathological conditions including protein oxidation, lipid peroxidation, DNA damage and cellular degeneration and these conditions have been implicated in the aetiology of diseases such as diabetes, cancer, Alzheimer and Parkinson disease, cardiovascular disease, aging process, arthritis and inflammation [4][5][6].

Interestingly, the body generates natural antioxidants to deal with the free radical load. There is always the need however to augment the efforts of these natural antioxidants with natural and synthetic products so as to support the body's defence, especially when they are overwhelmed by the load of free radicals generated. However, the continuous use of synthetic antioxidants from pharmaceutical stores has over the years post high state of panic following scientific assertions on their side effects [7]. This is not to completely assert that natural antioxidants are entirely risk free but since they are natural, there is a general believe of their safety over the synthetic ones [8]. Also, the accessibility and affordability of natural antioxidants is another strong reason for increased utilization of natural antioxidants over the synthetics. In Africa, poverty is a major problem. People rely more on cheaper options and tend to avoid expensive pharmaceutical products except when it has become obviously unavoidable. Hence this research tends to put the use of this plant product in better perspective.

In folkloric medicine which our ancestors practiced, plant materials are naturally grinded or chopped into smaller pieces and soaked in liquids which serve as vehicles for ingestion of the extracts. Sometimes the liquid is water, most of the other times, it is an alcohol. People are told to shake vigorously, allowed to settle, and take

shots of different sizes at different times of the day. This has been the practice over the years and Africans believe in its efficacy strongly. Our grandparents that lived most of their lives with this type of practices lived long, arguably longer than the present generation of Africans, hence the need to revisit this relatively declining practice.

Vernonia amygdalina (bitter leaf) has been a functional food plant. Reports show it is enriched with phenolic compounds like phytol, vernodalol, vernodalin, ascorbic acid, luteolin and luteolin-7-O-glucosides among others [9]. The method of extraction and type of solvent used are parts of the factors that determine the quality and quantity of the recovery yield and total phenolic content of the extracts [10]. Among these extracting solvents is water, ethanol, methanol, acetone, or their mixtures. Thus, the aim of this study is to evaluate the effect of different extracting solvents (water and methanol) on the recovery yield and total phenolic content of *V. amygdalina* leaf. Apart from type of solvent used, recovery of extract from plant matrix also depends on the temperature, pH, nature of the plant matrix, extraction time, and extraction method used in extraction [11]. These are however not our concern in the present study. *Vernonia amygdalina* has been a plant of choice for the treatment or management of several ailments, including wound healing [12][13], malaria [14], diabetes [15][16] and cancer [17]. Different parts of the herb have been used over the years all over Africa including the leaves, the root and the stem. People even use the stem as chewing stick up till today. This study was carried out on the leaves. Different methods have been used in ingesting the leaf of *Vernonia amygdalina*. Some squeeze it and drink the liquid extract. This might represent crude aqueous extraction. Other cut the leave and insert into a bottle of ethanol and drink after bouts of thorough shaking. This may represent ethanolic or methanolic extraction as the case may be. It is also highly nutritious. God'swill et al., [18] reported that the leaf of *Vernonia amygdalina* have a high content of carbohydrates, crude fat, crude protein, crude fibre, as well as a flavour which may enhance savoury and

palatability of food. This may explain the wide acceptability of bitter leaf in folkloric medicine to prevent or slow down the progress of various oxidative stress-related diseases.

The phenolic content in the leaves which indicates its antioxidant properties are utilized in the prevention of chronic diseases such as diabetes, cancer, heart diseases [19]. The antioxidant properties of *Vernonia amygdalina* have been linked to its polyphenolic content. Generally, the medicinal plant with more amounts of these antioxidant compounds could be more potent in scavenging free radical and ameliorating their pathological effects in the body. Thus, the aim of this research was to compare the antioxidant properties of *Vernonia amygdalina* in two commonly used extracting solvents (water and methanol).

Water is a chemical compound and polar molecule, which is liquid at standard temperature and pressure. It has the chemical formula H_2O , meaning that one molecule of water is composed of two hydrogen atoms and one oxygen atom. Water is present everywhere on earth and is required for sustenance of life. About 70% of the Earth's surface is covered by water [20]. An important feature of the water molecule is its polar nature. The water molecule forms an angle, with hydrogen atoms at the tips and oxygen at the vertex. Since oxygen has a higher electronegativity than hydrogen, the side of the molecule with the oxygen atom has a partial negative charge. The charge differences cause water molecules to be attracted to each other and to other polar molecules. Water is therefore a good solvent due to its polarity and practically the most used solvent in everyday life. The solvent properties of water are vital in biology, because many biochemical reactions take place only within aqueous solutions. When an ionic or polar compound enters water, it is surrounded by water molecules. The relatively small size of water molecules typically allows many water molecules to surround one molecule of solute. In general, ionic and polar substances such as acids, alcohols, and salts are easily soluble in water, and nonpolar substances such as fats and oils are not. Nonpolar molecules stay together in water because it is energetically more favorable for the water

molecules to hydrogen bond to each other than to engage in van der Waals interactions with nonpolar molecules [20].

Methanol is the simplest member in the alcohol family. It has a molecular formula of CH_3OH , and the molecular weight is 32 g mol^{-1} . Methanol is an extremely light, flammable, volatile, and colorless liquid. It has a characteristic smell and a burning taste. Melting point of methanol is -98°C , and the boiling point is 65°C . Methanol reacts with oxygen to produce carbon dioxide and water. It is naturally produced by anaerobic respiration of some bacteria varieties. It is industrially produced from fossil fuels such as natural gas or coal. Methanol is highly used as a solvent in laboratories for dissolving polar solutes. It is also used as a fuel in vehicles, antifreeze in car radiators, and as a denaturant. Methanol is highly toxic [21].

2 Methodology

2.1 Collection and authentication of plant material

The leaves of *Vernonia amygdalina* used in this study were obtained from Ekeoma market in Elele Rivers State and identified by Dr. Ekeke Chimezie, Department of Plant and Biotechnology, Faculty of Biological sciences, University of Port Harcourt, Nigeria with Herbarium number UPH/P/075.

2.2 Preparation of plant materials

The leaves were plucked and removed from their stalks. Leaves were then air dried for 4 weeks under room temperature. Dried samples were then ground into powder mechanically, using manual grinder.

2.3 Extraction Method

Four hundred (400g) of plant powder was soaked in 1000ml of distilled water and methanol for aqueous and methanolic extracts respectively and kept in a container for 24 hours. The mixtures were then vigorously shaken intermittently for additional 2 hours, to allow complete extraction. The resulting mixture was rapidly filtered using Whatman no. 1 filter paper to obtain a homogenous filtrate. This filtrate was then concentrated in vacuum at low temperature (37-

40°C) using a rotary evaporator. The concentrates were allowed open in water bath (40°C) for complete dryness yielding 30g each of brown gummy substance. The extract was later reconstituted in distilled water at a concentration of 1g/ml before administration. The extract was then refrigerated at 2-8°C until use. The reference doses of *Vernonia amygdalina* used in this experiment were 200mg/kg and 400mg/kg for low and high doses respectively. 1g of the extract of *V. amygdalina* was dissolved in 10ml of water to get the stock solution of 10mg/ml

2.4 Preparation of mercury chloride stock solution

Mercury chloride used for this study was of analytical grade. 0.5g of mercury chloride was dissolved in 125ml of distilled water yielding 4mg/ml (stock solution).

2.5 Experimental animals

Thirty male Wistar rats weighing between 180-200g were used for this study. The animals were purchased from the animal house of University of Port Harcourt. They were housed in standard cages and left to acclimatize for seven days under natural conditions in the animal house before the commencement of the experiment. They were fed rat chow and tap water ad libitum.

2.6 Experimental design

Thirty male Wistar rats weighing between 180-200g were used for this study. The rats were divided into 6 groups A, B, C1, C2, D1 and D2 of 5 rats each. Rats in group A received only food and water. All rats in groups B, C1, C2, D1 and D2 received mercury chloride (HgCl_2) at 0.5mg/kg/bw intraperitoneally two times in a week for 2 weeks according to the method reported on National toxicology program [22] (modified) to induce toxicity. Group B served as the positive control and received no treatment with *V. amygdalina*. Groups C1 and C2 received 200mg/kg per body weight of aqueous and methanolic leaf extracts of *Vernonia amygdalina* respectively while Groups D1 and D2 received 400mg/kg per body weight of aqueous and methanolic leaf extracts of *Vernonia amygdalina* respectively. All *Vernonia amygdalina*

administration was done orally using a gavage and lasted daily for 14 days. On the 15th day the rats were subjected to the neurobehavioural tests (hanging wire test), after which they were sacrificed through cervical dislocation. The brains of the rats were harvested. Four of the brains were immersed into phosphate buffer solution at the rate of 1:4 for antioxidant studies. They were then homogenized and centrifuged at 10000rpm to separate the supernatant from the residue. The supernatant was used for the antioxidant assay. Antioxidant parameters tested include lipid peroxidation (MDA), super oxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT). The remaining brains from each group were fixed in 10% formal saline for histological studies using H&E procedure.

2.7 Histopathological Studies

After 48 hours of fixation, the brains were removed and the cerebellum excised from each one for histological assessment using H&E.

Dehydration: Tissues were placed in ascending grades of alcohol (70%, 80%, 90%, and absolute).

The time for each grade of alcohol is one hours and in absolute (100%) it is put twice, one hour each time.

Clearing: Xylene was used to remove alcohol which itself removed water from tissue because paraffin wax used in embedding is not miscible with alcohol. The tissue was passed twice for one hour each time.

Embedding: The tissues were embedded in molten paraffin wax at constant temperatures of 36-60°C in an oven of paraffin bath, changing it four times, 1 hour for each change. **Block making:** Metal blocks were taken and filed with paraffin wax and tissues were placed in it immediately with forceps, the face to be cut facing downward. When the paraffin cools, a thin scum of solid paraffin is formed on the bottom of the block which is now immersed in water. Solidifies and was removed, ready for sectioning.

Sectioning: The section was cut thin to 5µm using a rotary microtome after excess paraffin wax was trimmed off.

Mounting of paraffin section: A slice of the section was taken and one side of the glass slide was made sticky by rubbing albumin of egg. The section was put in the centre of the slide so that the section starts floating. The section was immersed in water bath,

keeping temperature between 50-55°C so that sections become straightened and wrinkles disappear. Water was drained off and the slide is put in an incubator so that the section is completely fixed on the slide and becomes dry.

Staining: Haematoxylin and eosin staining technique: Procedure for H&E stain as described by Drury and Wallington [23] was adopted. The sections were dewaxed in two changes of xylene for 2 minutes each, rehydrated in descending grades of alcohol, 100%, 95%, 90%, 70%, 50% ethanol for 2 minutes each, rinsed in distilled water, stained in haematoxylin for 10-15 minutes, washed well in running tap water for 2-3 minutes

and examined microscopically to confirm sufficient degree of staining. Tissue differentiated in 1% HCL acid for a few seconds to remove excess stain. Sections were then washed in running tap water for 15 minutes to regain the blue colour and then stained in 1% aqueous eosin for about 5 minutes. Surplus stain was washed off in running tap water and mounted in distrene plasticizer xylene (DPX) using clean glass cover slide. Tissues were then focused under Leica research light microscope and photomicrographs taken from each group and labelled using Microsoft power point (Plate 1-3).

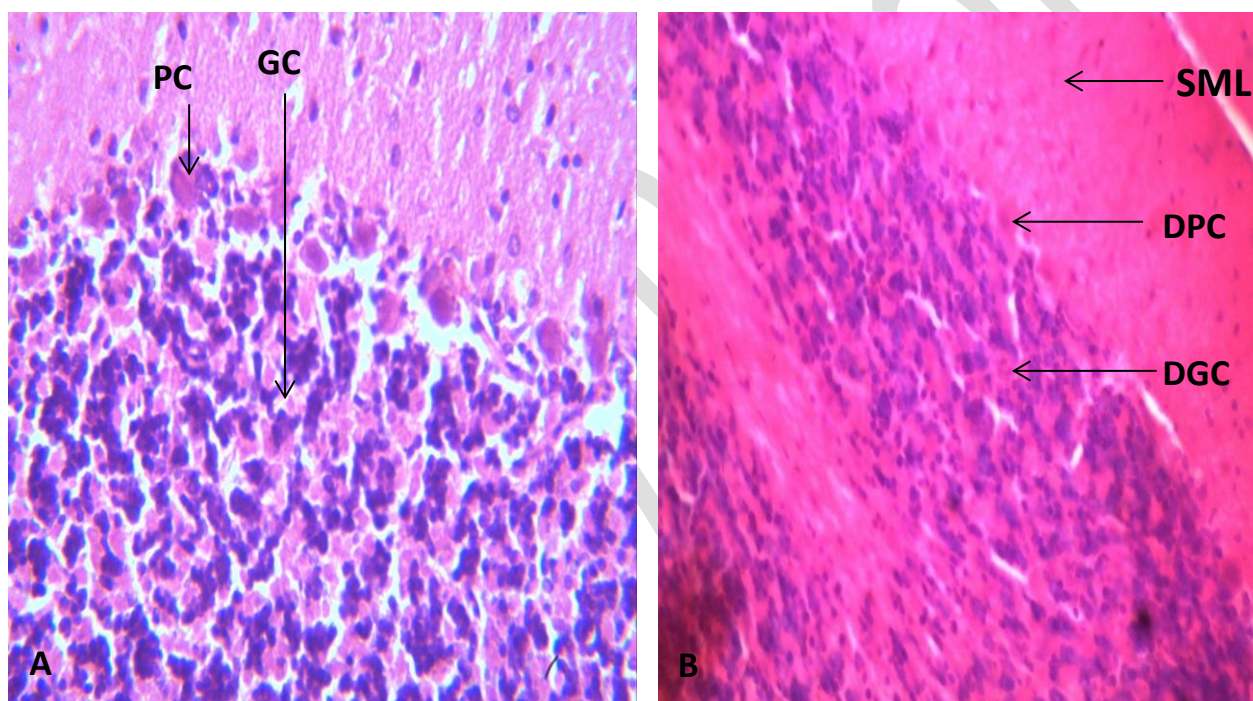


Plate 1: Representative photomicrograph of rat cerebellum. Group A shows normal cerebellar cytoarchitecture with normal purkinje cell (pc) and granular cell (GC). Group B shows scanty molecular layer (SML), dying purkinje cells (DPC) and dying molecular cells (DMC).

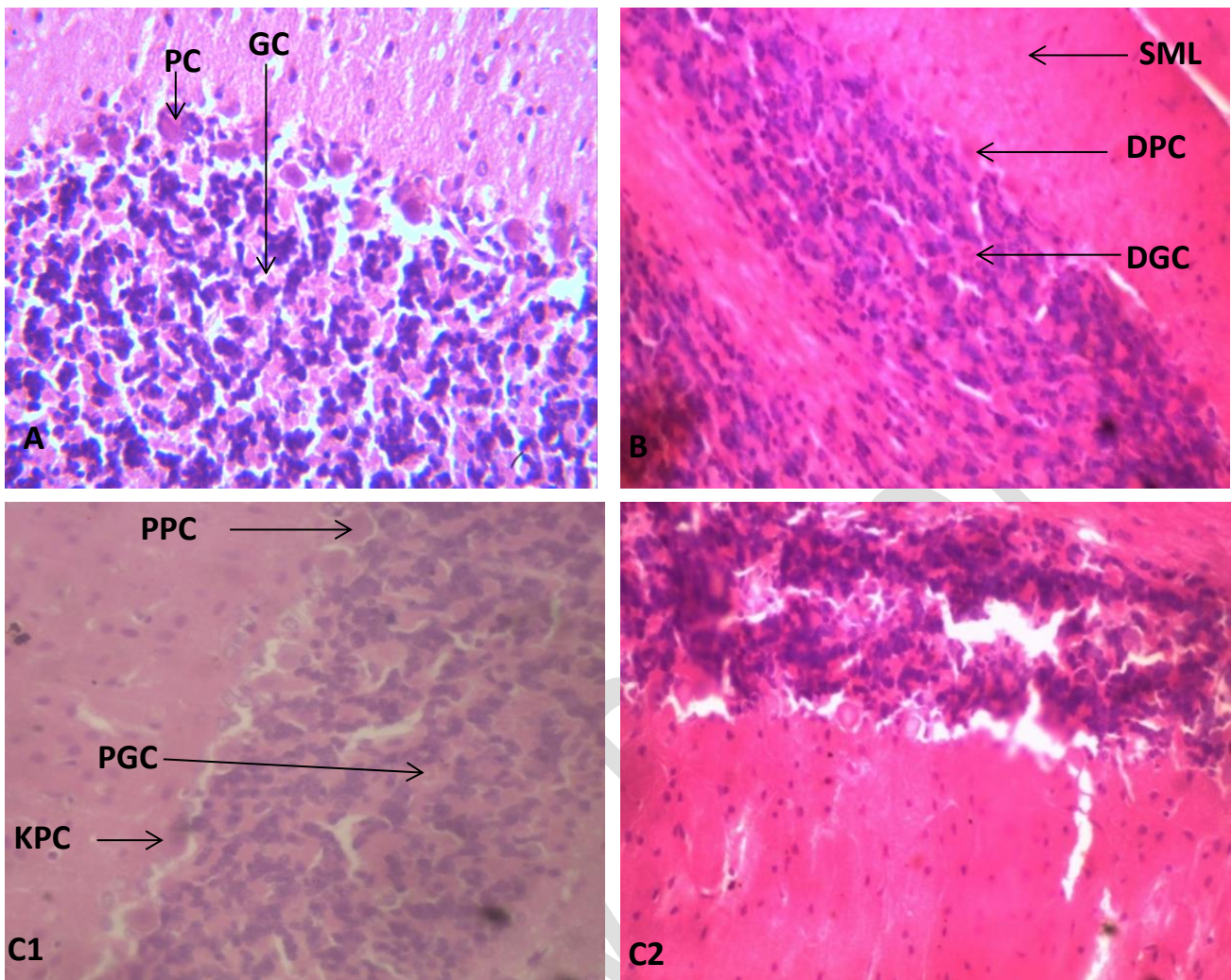


Plate 2: Representative photomicrographs of rat cerebellum comparing the cerebellar of the C-groups with those of the control groups A & B. Group C1 shows Pyknotic purkinje cells (PPC), karyorrhetic purkinje cells (KPC) and pyknotic granular cells (PGC) and C2 scanty purkinje cell layer (SPCL) and many necrotic purkinje cells (NPCs).

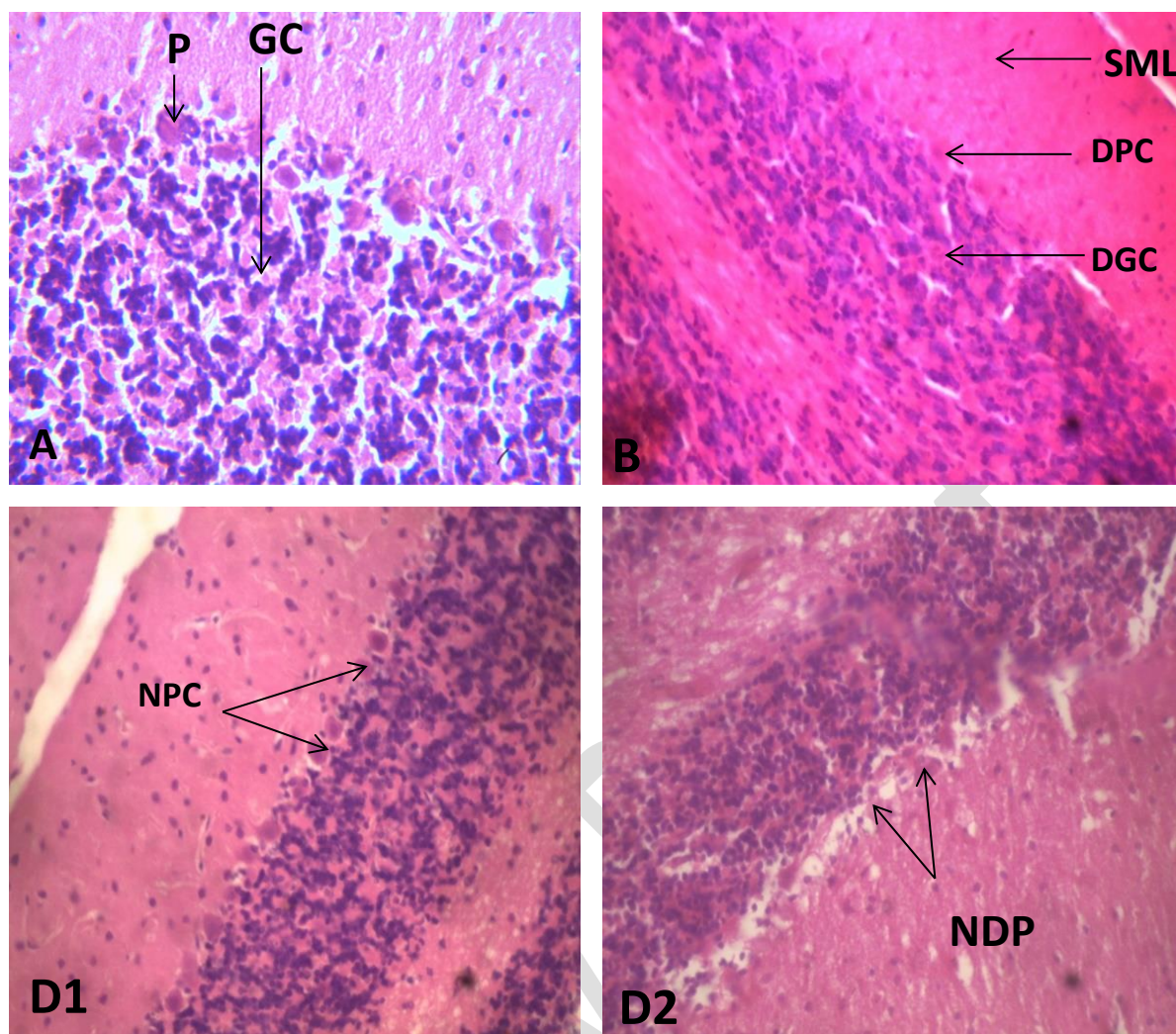


Plate 3: Representative photomicrographs of rat cerebellum comparing the D-groups (D1&D2) with the control groups A&B. Group D1 shows normal purkinje cell (NPC) but D2 shows numerous dying purkinje cells (NDPc)

2.8 Anti-oxidant assay

Brain samples from each group were taken for anti-oxidant tests to examine the activities of Superoxide dismutase (SOD), Catalase (CAT), Reduced glutathione (GSH) and Malondialdehyde (MDA). The brain samples were collected and weighed. Phosphate buffer solution was prepared and poured into the specimen bottle containing each brain sample for homogenization using a homogenizing machine. The sample was centrifuged with cold centrifuge at 10,000 rpm (rounds per minute) and the supernatant was collected. The rest of the assay was done using the supernatants.

The protein concentrations of the samples were determined by the Biuret method as described by Gornal et al., [24] which has been modified by the

addition of potassium tartarate to prevent precipitation of copper ions (Cu^{2+}) as cuprous oxide. This was carried out by the method of Varshney and Kale [25]. This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde (MDA) – an end product of lipid peroxidation. The level of SOD activity was determined by the method of Misra and Fridovich [26]. The ability of SOD to inhibit the auto-oxidation of epinephrine (adrenaline) at pH of 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide (O_2^-) radical generated by the xanthine oxidase reaction caused by the oxidation of adrenaline to adrenochrome and the yield of adrenochrome produced per O_2^- increased with increasing pH [27] and with increasing concentration of adrenalin. The level of reduced glutathione

(GSH) was estimated using the method of Beutler et al., [28]. The reduced form of glutathione comprises in most cases the bulk of cellular non-protein sulfhydryl groups. This method is hereby based on the development of a relatively stable yellow colour when 5', 5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with reduced glutathione, 2-nitro-5-thiobenzoic acid possesses a molar absorption which was read at 412nm in a colorimeter.

2.9 Neurobehavioural Studies

The hanging wire test was carried out on the rats the day after the last administration for cerebellar function. The fore limb hang test uses a wire grid system to non-invasively measure the ability of rats to exhibit sustained limb tension to oppose their gravitational force. The procedure measures the fore limb hang time in seconds. The fore limb hang test is performed to demonstrate neuromuscular impairment and motor coordination [29]. It is an efficient and reliable outcome measure for the evaluation of effects of therapeutic compounds on muscle strength. However, due to the nature of the test, it is not possible to relate the outcome to a sole neuromuscular defect, or muscle. The test is easy to perform and inexpensive, although animals need to be supervised at all times, making the number of animals which can be simultaneously analysed relatively small. Rats are clearly willing to perform the test since behaviour indicates that they do not want to fall off the grid [29].

2.10 Statistical Analysis

Data obtained were analysed using students T test with Microsoft excel and expressed as Mean \pm SD. Differences between means were regarded significant at $P < 0.05$.

3 Results

Table 1 shows that the aqueous extract of bitter leaf had higher quantity of flavonoids and alkaloids while methanol had higher saponins. Both had similar concentrations of phenols, tannins, steroids and glycosides.

Table 1: Result of Qualitative Phytochemical Analysis of Vernonia amygdalina

PARAMETERS	AQUEOUS	METHANOLIC
Saponins	++	+++
Flavonoids	+++	++
Alkaloids	+++	++
Phenols	+	+
Tannins	+	+
Steroids	+	+
Glycoside	+	+

NB: +++ Highly present, ++ Moderately present, + Low, - Not present

From table 2, it is seen that the methanolic extract had higher phenol, tannins and steroid counts compared to the aqueous extract.

Table 2: Result of Quantitative Phytochemical Analysis of Vernonia amygdalina

PARAMETERS	AQUEOUS	METHANOLIC
Saponins	7.29	9.03
Flavonoids	6.58	4.38
Alkaloids	9.26	7.32
Phenols	0.78	0.95
Tannins	0.13	0.61
Steroids	0.15	0.28
Glycoside	0.41	0.37

The results of the phytochemical analysis above show that considering phenol and flavonoid content which represent the antioxidant properties of a plant material, water extracted more flavonoids while methanol extracted more phenols. But from a general consideration, water gave more antioxidant phytochemicals (flavonoids plus phenol) than methanol.

The results of the antioxidant studies presented in Table 3 indicated that malondialdehyde (MDA) levels were increased across all experimental groups compared to the control. However, only group B was statistically significant. Group B received 0.5mg/kg/bw of mercury chloride intraperitoneally twice a week for the 2 weeks of the experiment without any treatment. However, a look at the treated groups C1 to D2 shows that the groups treated with the methanolic extracts had correspondingly more reduced levels of MDA both in the low dose and high dose.

Superoxide dismutase (SOD) was reduced in all the experimental groups B, C1, C2, D1 and D2 compared to the control group A. The differences however were not statistically significant except in groups B and C1. Here again, it was observed that the C2 and D2 groups which received the methanolic extracts of bitter leaf had lesser depletion of SOD compared to the aqueous groups C1 and D1. Typically, under oxidative stress, there is depletion of SOD level with a corresponding rise in MDA levels. Catalase (CAT) showed significant reduction in the experimental groups B without bitter leaf intervention, as well as in groups C1 and D1 which received the aqueous extract of the plant. However, catalase levels in groups C2 and D2 were statistically similar to those of the control group. Glutathione peroxidase (GSH) was highly significantly reduced in all experimental groups when compared to the control.

Table 4 shows the results of the hanging wire test carried out on 5 rats in each of the 6 groups used for the experiment. INITIAL represents the data collected prior to experimentation (after acclimatization) while FINAL represents data collected at the end of the experimental period of 14 days. There was no statistically significant difference between INITIAL and FINAL for the control group A which received only food and water.

However, following exposure to mercury chloride, there was a highly significant difference in the means of the INITIAL AND FINAL data for group B which received mercury chloride only without treatment. When treatment of V. amygdalina was administered a little improvement was observed across the groups. But these were not statistically significant. It is therefore difficult to ascertain the impact of the different extracts on improving suspension time in the rats and hence neuroprotection.

Table 3: Result of Antioxidant Studies.

GROUP	MDA	SOD	CAT	GSH
A	0.32±0.16	0.40±0.17	3.23±0.58	7.43±0.77
B	0.55±0.10*	0.15±0.07*	1.74±0.36*	3.72±0.54**
C1	0.46±0.11	0.20±0.09*	1.93±0.38*	3.80±0.30**
C2	0.43±0.06	0.31±0.08	2.59±0.70	4.30±0.62**
D1	0.45±0.15	0.28±0.06	2.04±0.24*	4.00±0.35**
D2	0.40±0.12	0.33±0.08	2.90±0.35	4.95±0.36**

Data is presented as Mean ± Standard deviation of 7 rats in each group.

*indicates statistical significance at P<0.05

**indicates high statistical significance at P<0.05

Table 4: Result of Hanging wire Test

GROUP	INITIAL	FINAL	P-VALUE
A	4.38±1.10	4.46±0.40	0.54249
B	4.20±0.65	1.14±0.25	0.00006
C1	4.36±0.45	2.14±0.45	0.00000
C2	4.27±0.40	1.83±0.58	0.00029
D1	4.19±0.50	2.43±0.86	0.00866
D2	4.20±0.45	2.05±0.37	0.00002

Data is presented as Mean ± Standard deviation of 4 rats in each group.

*indicates statistical significance at P<0.05

**indicates high statistical significance at P<0.05

Table 5: Comparing the results of the Hanging wire Test across groups.

GROUP	A	B	C1	C2	D1	D2
INITIAL	4.38±1.10	4.20±0.65	4.36±0.45	4.27±0.40	4.19±0.50	4.20±0.45
P-VALUE		0.57	0.92	0.67	0.51	0.49
FINAL	4.46±0.40	1.14±0.25	2.14±0.45	1.83±0.58	2.43±0.86	2.05±0.37
P-VALUE	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Data is presented as mean ± standard deviation of 7 rats in each group.

*indicates statistical significance at P<0.05

**indicates high statistical significance at P<0.05

Table 5 presents the results of the hanging wire test, comparing the INITIAL data across the groups as well as the FINAL data across the groups. The initials were all similar and no statistical difference was observed. This implies that at the commencement of the experiments the rats were healthy and performed uniformly in the test irrespective of the group. However, at the FINAL stage, the see highly statistically significant differences between the control group and the rest of the experimental groups. It means that exposure to mercury chloride grossly affected the animals limb strength and suspension time. This could imply cerebellar dysfunction.

4 Discussion

Our result showed that the extracts of bitter leaf used for this research showed antioxidant properties in a dose dependent fashion. This is inferred following our results which reveal that for all the tests carried out, the D groups which received 400mg/kg/b.w of *Vernonia amygdalina* performed better than the C groups which received 200mg/kg/b.w. This is supported by the work of Aruoma et al., [30] which reported that the efficacy of bitter leaf increased with increasing doses. The level of lipid peroxidation (MDA) decreased in the experimental groups C1 to D2 following administration of the different extracts of *Vernonia amygdalina* compared to group B. It also showed that the values for MDA in the D groups were higher than those of the C groups. This represents a dose-dependent pattern of action. It means that the higher the dose consumed, the better the result expected. This is supported by the work of Udochukwu et al., [18] which showed that both aqueous and methanolic extracts of VA prevented lipid peroxidation. Aguwa et al., [31] also reported the

antiperoxidative effect of leaves of *Vernonia amygdalina*. Another study by Nwanjo [32] on diabetic rats showed that the aqueous extracts of VA decreased the levels of serum malondialdehyde.

Our result in table 3 however shows that the aqueous extract of *Vernonia amygdalina* showed better antioxidant properties than the methanolic extract. These also were shown to act in a dose-dependent fashion. The two extracts reduced the extent of lipid peroxidation. This was evident in the decreasing levels of malondialdehyde (MDA) in the treatment groups C1 to D2 when compared to group B which received no bitter leaf extract. Furthermore, the levels of the antioxidant enzymes which were significantly depleted in the experimental group B showed a rise with *Vernonia amygdalina* administration in a dose-dependent manner. We are therefore convinced by these results in line with the finding of other researchers that *Vernonia amygdalina* is a potent antioxidant. But we noticed from our result that the aqueous extract conferred better resistance to oxidative stress than the methanolic extract. Levels of MDA were the least in the aqueous groups C1 and D1 compared to their methanolic counterparts (C2, D2). It is reported that the antioxidant potential of the plant material usually appears to correlate with the phenolic content. Differences in the structure of phenolic compounds also determine their solubility in solvents of different polarity. Therefore, type of extraction solvent as well as the isolation procedures may have a significant impact on the yield of extraction polyphenols from plants material [33][34]. Reports also show that plant phenolics are major group of compounds acting as primary antioxidants or free radical scavengers [19].

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the level the body's natural antioxidant defence mechanisms can cope with, causing damage to macromolecules such as DNA, proteins and lipids [35]. Although many synthetic drugs exist which are used as antioxidants, reports have shown that the users are also exposed to other dangers. Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are suspected to be tumorigenic. This is the reason for preference of natural alternatives, and this research goes ahead to confirm that bitter leaf is one of such natural alternatives.

The results of our research as presented in table 3 shows that both extracts used for this study (aqueous and methanolic) showed antioxidant quality. In group B which did not receive any treatment following mercury chloride intoxication, there was significantly higher levels of MDA which is an indicator of lipid peroxidation. The rise in this marker indicates increase in oxidative radicals [31]. Reduced glutathione (GSH) is an endogenous antioxidant which plays a vital role in the detoxification of xenobiotics and scavenging of free radicals or reactive oxygen species (ROS) in cells [31]. A decline in cellular level has been considered to be indicative of oxidative stress. The decrease in this endogenous antioxidant in our experimental groups B to D3 supports the presence of oxidative stress in our experimental animals. Also reduced were levels of catalase (CAT) and super oxide dismutase (SOD). This trend (increase in MDA levels with corresponding decrease in SOD, CAT and GSH) leave us with no doubt that mercury chloride administered twice a week intraperitoneally at 0.5mg/kg body weight of rat induced oxidative stress in the rats in the experimental group when placed in the light of the results of the rats in the control group. Also the result of the biochemical analysis presented in table 3 shows that the rats in the experimental groups B to D2 which received mercury chloride only were under oxidative stress. We therefore put forth that the result of our study adds to the existing literature that exposure to mercury chloride is toxic to the brain of Wistar rats. These

evidences lend support to our results which shows that mercury chloride not only elicited oxidative stress in the rats, but also led to cell deaths and cytoarchitectural distortion of the tissues. These toxicities were expressed as the rats performed poorly in the neurobehavioural tests where they actually performed relatively better before exposure to mercury chloride. Reports from the Agency for toxic substances and Disease Registry [36] shows that exposure to mercury causes a wide variety of cognitive, personality, sensory, and motor disturbances. These impairments point to pathology of the cerebellum among others. They also reported symptoms such as tremors, nervousness, memory loss and neuromuscular changes including muscle atrophy and weakness [36].

Exposure to mercury is detrimental to the good health and well-being of living organisms. Mercury toxicity still remain a public health issue across different countries in the world especially the developing nations. In most of these nations, regulations are not properly followed up to ensure that industrial wastes are properly disposed of. Studies have shown that the commonest source of ingesting mercury is through consumption of sea food especially fish. Mercury is used as fungicides, antiseptics, preservatives, electrodes and reagents [37]. Mercury is also a known Hazardous Air Pollutant (HAP). Natural sources include geothermal venting, evaporation, vegetation release, wildfires and soil out-gassing [38]. Although the doses of mercury chloride acquired through fish consumption may not lead to instant toxicity, mercury is known to accumulate in the human body and so may manifest its toxic effects after many years. The result of this research shows that mercury chloride as low as 0.5mg/kg b.w of rat administered intraperitoneally twice a week produced signs of toxicity on the rats. This was manifested through several physical signs of weakness including reduction in size, reduced food consumption and reduced general agility. Some of the rats even died in the course of the experiment.

Therefore, toxicity might predispose to chronic ailments over time and eventually lead to reduced life expectancy. The toxicity of mercury chloride

is no longer in doubt as several researchers have reported the toxic effects of mercury chloride on different organs and systems of the body, including the nervous system. Barregard, [39] reported on the toxic effect of mercury on erythrocyte and leucocyte count; Rao et al., [40] on the liver; Hallee, [41] and Goyer, [42] on the lungs; Rice et al., [43] and Clarkson, [44] reported on the gastrointestinal tract, including bleeding gums, loosening of teeth, mouth and throat ulceration, excessive salivation and foul breath; Pambor et al., [45] and Goh, [46] reported on erythemas and dermatitis following contact with skin; Thurtson et al., [47] and Altman, [48] reported on the heart and cardiovascular system; Booth, [49] reported skin rashes, dim vision and clamminess; Rao & Sharma, [50] and Baltimore [51] reported on menstrual disturbances and pregnancy complications following exposure to mercury through different routes. Similarly, Abubakar [52] reported that mercury intoxication reduces the concentration of copper in the brain. Zucconi et al., [53] in the same vein reported that decrease in copper concentration in the brain can interfere with the modulatory role of dopamine receptors and consequently lead to motor function deficits similar to what is seen in patients of Parkinsonism.

The poor result of the hanging wire test presented in Table 4 reveal that the rats had muscle weakness and so could not hold up long enough on the hanging wire. The hanging wire test was carried out to test for muscular strength and by extension cerebellar function. In this case the animals were suspended on a hanging wire using their forelimbs. The rationale is to determine how long they can suspend there which by extension is an indicator of muscle strength and motor activity. The results were compared with those taken before mercury chloride and *Vernonia amygdalina* administration. So, the initial records are here referred to as INITIAL while the final data collected at the end of the experiment were referred to as FINAL. Our result as presented in tables 4&5 shows statistically significant reduction for FINAL when compared to INITIAL in all the experimental groups (B, C1, C2, D1, & D2). In the control group however, there was no

statistically significant difference between INITIAL and FINAL. In fact, FINAL was even higher than INITIAL. This could be owing to the fact that the rats were already aware of the procedure and could device means of lasting longer than at their first exposure. The reduction in the FINAL for the experimental groups is an indication of weakness as well as reduced muscular strength. This result is supported by the histology results which show varying degrees and stages of necrosis following mercury chloride intoxication. It has been shown that exposure to high concentration of mercury vapor causes tremor initially affecting the hand and then spread to the other part of the body [54][55]. However, we observe that the groups treated with the aqueous extract of *Vernonia amygdalina* had longer time of suspension compared to those that received methanolic extracts. This was evident in both the C and D groups. Based on the result of the neurobehavioural studies, we could infer that the aqueous extract of *Vernonia amygdalina* conferred more protection on the cerebellum leading to a better retention of the functions sub served by it.

Histopathological assessment of the cerebellum showed various degrees and stages of cell death, distortion of the Purkinje cell layer, infiltration of cells in the granular cell layer and general disorientation of the architecture of the Purkinje cell layers. There was sparse distribution of Purkinje cells of the Purkinje cell layer especially in group B which received mercury chloride without treatment. The Purkinje cell is the sole motor output of the cerebellar cortex. Reduction in its number, size or efficiency could lead to interferences with the motor functions such as loss of fine movement, loss of grasping, maintenance of equilibrium and loss of regulation of muscle tone. Moreover, it has been shown that neuronal degeneration in the cerebellum can affect the level of copper concentration in the cerebellum and this can affect the action of the neurotransmitter, dopamine which is very crucial in motor activity. This is because copper serve as a modulator for the neurotransmitter, dopamine and a decreased in copper level may reduce dopamine activity. Mutter *et al.*, [56] had reported decrease in dopamine concentration in the

cerebellum of mice exposed to cadmium chloride for a week which resulted in motor function disorder. It has been shown that mercury intoxication does not show any significant changes in the granular layer of chicken treated with mercury chloride in their food [57][58]. This is not in agreement with the findings of the present study which showed infiltration of granular cells and other forms of cytoarchitectural degeneration. Fuyuta et al, [59] reported abnormal cytoarchitecture of the brain in infants prenatally exposed to mercury. Mercury is known to bind to microsomal and mitochondrial enzymes, resulting in cell injury and death [60]. This could explain the poor performance recorded the hanging wire test for cerebellar function carried out after exposure to mercury chloride when compared to the results gotten prior to exposure to mercury chloride.

5 Conclusion

Vernonia amygdalina confers protection on the human brain against oxidative stress. For raw consumption, aqueous extract is recommended as it is potent and readily available. For other industrial processes, water and methanol are equally potent, depending on the purpose of the procedure. *Vernonia amygdalina* of the variety found in the southern part of Nigeria can be fabricated into orthodox supplements consumed regularly for its antioxidant properties. Conscious consumption of raw bitter leaf and in form of juice as a natural source of antioxidants is therefore highly recommended. Anambra people if Igbo extraction as well as other parts of south east Nigeria have a popular bitter leaf soup. This should be encouraged as a regular part of our diet. We also advocate planting of this highly medicinal herb around every household in Nigeria and around the world. This study also goes ahead to prove that laboratory phytochemical analysis is not enough to confirm the efficacy of a herb until it has been subjected to direct experimentation as was the case in this study. We recommend the conscious consumption of bitter leaf and soups and in form of juice as a natural source of antioxidants. We also recommend that further researches be carried out with several solvent combinations to

know the combination that can give the best antioxidant yield.

6 Declarations

6.1 Acknowledgement

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6.2 Ethical Approval

Due ethical approval was gotten from the research ethics committee of the Faculty of basic Medical Sciences, Madonna University Nigeria.

6.3 Competing Interests

The authors declare that there is no conflict of interest.

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Will be updated in the final version.

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